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NEW POSSIBLE METHODS OF MAKING FLUORESCENCE-MARKED PROTEINS

by

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Besides the usual sulfochlorides ~~we also~~ found several fluorescent dyes containing sulfo groups to proteins. The most favorable conditions for this were determined, and the marked proteins were checked for purity by electrophoresis. The fluorescence spectrum of the dyes and a few conjugates were measured. By means of protein markings of different colors the possibilities for diagnostical, immunological and histochemical applications have been extended and improved. The method described is a rather simple way of making fluorescin marked proteins.

The method of Coons[1,2] for making proteins visible by binding to fluorescin, has found extensive application for the marking of antibodies and antigens:

The places where the antibodies are made are better localized[3,4] and the distribution of foreign antigens in the organism can be followed optically[5-7]. Microorganisms could be recognized with speed and certainty by means of fluorescent antibodies[2,8-10]. In Proteus bacteries it was found that the protein of L-forms and of the belonging bacteries is largely identical[11]. Connections between specific protein structures of liver cells and readiness of cancer formation gave rise to interesting ideas[12]. Ferments[13] and hormones[14] were found in tissues by fluorescence serology, and it was even possible to make virus antigen possible in infected cells[15-17]. A survey of methods and results was given by Coons[18], Poetschke and co-workers[19], and Mayersback[20-21]. Making fluorescin isocyanates according to the method of Coons[2,19] is complicated and takes

much time and some practice. Besides the yellow-green color is little different from the proper fluorescence of the cells. In spite of many improvements in methods[22,23] working with fluorescin isocyanate was often not satisfactory.

Therefore it was tried to bind other fluorescence dyes to proteins. Creech and Jones[24] already used β-anthrylisocyanate. Clayton[25] tried 1-dimethylamino-5-naphthalinsulfochloride. He was the first one to try to make several antigens visible at the same time by using marked antibodies with different colors. Silverstein[26] made rhodaminisocyanate. Because of various difficulties and disadvantages however none of these methods were further developed.

Therefore we have also tried to bind various fluorescin dyes to proteins with a reaction which can easily be accomplished, in order to make this valuable method also suitable for bacteriological or immunological routine investigations[27]. The reaction with sulfochloride was shown to be very suitable, because a few fluorescent dyes with sulfo groups are already commercially available or can easily be made.

At the same time and independently Chadwick, McEntegard and Nairn[28] published on the subject of successful protein markings with "Lyssamine Rhodamine B 200". This dye is identical with sulforhodamin B which we have also used.

Materials and Methods

The following dyes were tried for their suitability as protein markers: Aizarin S, Brillantsulfoflavin, Geranin, 3-hydroxypyren-5.8,10-trisulfon acid, Sulforhodamin B, Thioflavin S, Thiazin red (all from "Bayer").

We made: Sulfofluorescin from 4-sulfophthalic acid and Resorcin. The 4-sulfophthalic acid was synthathized according to l.c.[29]. Sulfo-acridinorange by sulforation of acridinorange with concentrated H_2SO_4 at 80°C. The resulting product is not completely sulfonated and difficult to isolate.

1-Dimethylamino-5-naphthalinsulfon acid was obtained by methylisation of simethylsulfate.

Fluorescent spectra: The spectra were obtained with the fluorescence accessory to the Zeiss spectrophotometer. The excitation wavelength was 364 mμ. Of all dyes 0.1 milli mole was solved in m/100 carboante buffer with pH 8.2. The comparison solution was chininsulfate. The calculation was done by means of a known absolute calibration curve for the chininsulfate.

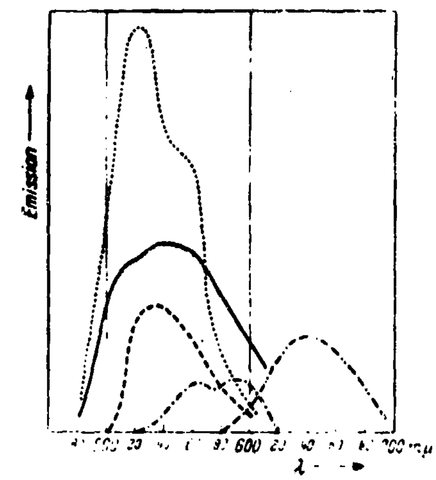

Fig. 1. Fluorescence spectra: ———— Brilliantsulfoflavin, ········ 3-Hydroxypyren-5.8.10-trisulfon acid, — — — — fluorescin, —·—·—·— acridinorange, x x x x x sulforhodamin B. All dyes 0.1 millimole in m/100 carbonate buffer pH 8.2. Exciting wavelength: 364 mμ.

Making the sulfochloride: About 1 gram of the sulfo dye concerned is ground up with a nearly stochiometric amount of phosphorpentchloride in a small mortar. After a few minutes the mixture is removed with about 5 ml. cold acetone and is filtered immediately in the cold into a polished reaction tube. Then a bit water free sodium sulfate is added. This solution can be kept for weeks in an xeiccator at low temperatures and away from the light.

Binding to proteins: The protein solutions that have to be marked are put together with 3 volumes of 0.2 m tris buffer (Tris (hydroxymethyl)-amonomethane with HCl at a pH of 9.0) in a short wide reaction glass, which has a stirring system. At a temperature of 0-2°C the solution of sulfochlorides is slowly added from a sucked out pipet (0.1 ml. in about 15 minutes). This pipet is best mounted in a fixed position so that the solution comes out under the surface of the reaction mixture. The regulation is accomplished with a pinch clamp on a rubber hose.

The solution is not allowed to be acid after addition of the sulfochlorides. Otherwise most dyes would precipitate, but a small addition of tris buffer will bring them in solution again.

About 30 μ mole sulfodye are combined with 100 mg. protein (determined with the biuret method). The mixture is stirred 2 to 3 hours in the cold and then immediately dialysed against m/15 NaCl in m/100 phosphate buffer with pH 7.4.

Example: 2 ml. of an 8% protein solution is diluted with 6 ml. trisbuffer. There is 160 mg. protein in the now 2% solution. 1 gr. hydroxypyrensulfon acid is ground up with 0.5 gr. PCl_5 and then taken up with 5 ml. acetone. Per milliliter there is now about 200 mg dye. Between 0.08 and 0.1 ml. is used, that is between 16 and 20 mg dye (1 μ mole = 0.525 mg).

Repeated binding to dye: It has been found that in ordinary use of fluorescein isocyanate only 3 to 6% of the possible conjugation places of the protein is bound to fluorescein[30]. Therefore it was tried to have it react repeatedly to isocyanate. This used to be accompanied by high losses of protein by precipitation, but that does not occur in our method. After a single treatment with a sulfochloride the solution is dialysed for about 24 hours against a large amount of fluid (stirring!). After this the volume of the eggwhite solution is measured and one tenth the volume of 2 m tris buffer with pH 9.0 is added. Now renewed treatment with sulfochloride can be undertaken.

Purification: For the removal of unbound dyes a sufficient dialyses can be recommended in any case. Usually 3 to 4 days with daily change of the dialisation fluid and continuous stirring will do. The time of dialyses can be reduced by treatment with active carbon. Per ml of mixture 50 mg of carbon powder is added and then the mixture is shaken strongly. Subsequently it is centrifuged at at least 3000 g. It is of great advantage to do all stages in a cold room.

Proteins are often unspecifically bound in small amount by cells and tissues, and this can already lead to annoying unspecific fluorescence of the object that has to be investigated. By adsorbtion of an organic powder this phenomenon can often be eliminated. Acetone dried powder from rabbit or rat liver is specially suitable for this (compare l.c[3]).

Extraction of unbound dyes with organic solvents, as proposed by Dineen and Ada[23] with acetic ester, does not always give the desired result. The distribution between the watery and the organic phase must in any case be carefully tried in advance for the dye concerned.

The protein dye compounds have the same maxima in the emission spectrum as the sulfon acids.

Often the eggwhite contents of the fluorescein marked protein solutions is too small. In these cases we have concentrated the solution again by means of narrowing dialyses containers (apparatus for concentration

o. liquors from the membrane filter company Göotingen. In earlier publications[19] we have written concerning practical methods for the gathering and preparation of the immunization proteins that have to be marked, and about color technique and fluorescence microscopy.

Control: Cattle albumen are bound to various sulfo dyes, as described. After dialyses and purification they are mixed with a solution of cattle γ-globulin. After addition of 0.01 ml of a 0.1% merthiolate solution per ml the mixture is stored at 37°C for 12 to 16 hours. Subsequently the proteins are separated by electrophoreses on paper strips. The strips are cut in half and one half is routinely colored with amido black. Of the other half an ultra violet photo print is made. Now only the marked protein band may be visible by fluorescence. Still present free dye comes mostly ahead of the albumen.

Results

Some of the dyes that were tried had too little fluorescence. Thiazin red and geranin fluoresce very weakly in the blue region (max at 416 mμ). The 1-dimethyl-5-naphthalin sulfon acid also had a much weaker emission than fluorescin. Surprisingly strong is the fluorescence of the hydroxypyrensulfon acid. The maximum radiation is at nearly the same wavelength as fluorescin, namely 524 mμ. In contradiction Emmart[31] found for fluorescin an emission maximum at 550 mμ, but he did not give the wavelength of the exciting light.

Changes in the pH values do not change these maxima. Only the intensity decreases rapidly at a pH of below 7. This can also clearly be seen in the adsorbtion spectra of fluorescin and other protein conjugates given by Emmart. On the basis of the spectra the hydroxypyrensulfon acid with yellow green fluorescence and the sulforhodamin B with red fluorescence seem to be the best dyes.

The reaction of the sulfochlorides with the proteins gave really stable compounds which were at least equal in quality as those obtained from the isocyanate. The protein properties of such conjugates seem not to be changed, as is proven by electrophoresis and animal experiments[28].

Hydroxypyrensulfon acid sulforhodamin B have very much different fluorescence. Both dyes can be bought commercially. It is easy to make the sulfochlorides and the compounds with the proteins. Therefore practically every laboratory will be able to make fluorescin marked proteins for the most different purposes.

Research with fluorescence marked antibodies. III Progress report.

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